Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 7760 - 7771
Received 16 February 2021; Accepted 08 March 2021.

In Vitro Analysis of Neuroprotective and Antioxidant Property of Sponge Extract

G.B. Priyadharshini[1], C. Jaynthy[2], G. Seghal Kiran[3], Joseph Selvin[4]
[1,2]Department of Bioinformatics, Sathyabama Institute of Science and Technology
[3] Department of Food Science and Technology, Pondicherry University
[4] Department of Microbiology, Pondicherry University

ABSTRACT

Neurodegeneration is a progressive loss of structural and functional properties which finally leads to death of neurons. The accumulation of high concentration of the reactive oxygen species (ROS) in brain cells leads to damage and apoptosis which finally leads to the neurodegenerative disorders like Alzheimer (AD), Parkinson (PD) and Huntington disease etc., thus neuroprotective activity plays an important role in brain, working as a defensive mechanism which can either be self-developed or can be obtained through medication. Natural marine products are a rich source of novel compounds that can alter mammalian neurological activity. Marine sponges are one of the most sustainable and efficient sources to explore when bio-prospecting for neuroprotective compounds that can play a vital role in medicine. The sponge protein from *Callyspongia sp*. was extracted using β- mercaptoethanol and triton X-100 method. The main aim of the study is to unravel that the extract form *Callyspongia sp*. exhibits an excellent wound healing activity and cell viability in L929cell lines.

Keywords
Neurodegeneration, Marine sponges, wound healing activity and cell viability

INTRODUCTION

Brain being the most important organ of the human body suffers from many problems among which the damages caused due to oxidative stress by reactive oxygen species was the most common. Oxidative stress was defined as the imbalance in the redox potentials in intra and extracellular which was caused by high concentrations of free radicals [1]. The frequently reported free radicals were found to be hydroxyl (OH*), super oxide (O^{2-}) and nitric oxide (NO*). Also hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) which were not free radicals by nature but were reported to generate the free radicals by chemical reactions. The oxygen related free radicals and the reactive oxygen species were produced mainly as the result of aerobic metabolism [7]. This oxidative stress can be triggered by high exposure to UV radiation, pollution and due to toxic agents [2, 3]. Once when the oxidative stress was achieved, the organism experiences an unrepairable damage which includes change in the redox potentials, early aging process, apoptosis and development of chronic disorders like cancer, neurodegenerative, rheumatoid arthritis, cardiovascular problems and diabetes, arthrosclerosis [5, 6].

The early evidence showed that oxidative stress is one of the major causes for the outset of various neurological disorders. The reactive oxygen species especially seen in the brain and neuronal tissues were found to be the by-products of the metabolism which were unique in the brain. The accumulation of these by-products serves as the source of oxidative stress and their related damages. Thus, the accumulation of the Reactive Oxygen Species (ROS) in brain triggers the high concentration of hydrogen peroxide (H_2O_2), Nitric Oxide (NO), Hydroxyl radical (OH^-), lipid peroxides and superoxide ions, which were highly toxic leads to cell damage and finally apoptosis thus giving rise to neurodegenerative disorders like Alzheimer (AD), Parkinson (PD)

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 7760 - 7771
Received 16 February 2021; Accepted 08 March 2021.

and Huntington (HD). Neuroprotective activity was the defence mechanism against the degenerative disorders in brain, which was highly significant [7].
Marine environment plays a key role in the field of search for drug targets that were responsible for numerous diseases. Among all other organisms in marine environment sponges were found to measure a rich source of secondary metabolites and proteins which have several biological activities especially neuroactivity and can be extracted easily [8]. Since phylum Porifera was identified to contain the basic chemical messengers such as diffusible amino acids glutamate and γ-amino butyric acid (GABA) and a short-lived diffusible gas, nitric oxide (NO) that were found in CNS of animals, plants and other invertebrates which can be useful in the treatment of neurodegenerative disorders [9]. The aim of the work starts with the sponge extraction which was followed by their test for activity against the free radicals such as Nitric Oxide, hydrogen peroxide, superoxide, hydroxyl radical and iron reducing property etc.,

MATERIALS AND METHODS

Collection and Extraction of Protein Sample from Marine Sponge

The sponge samples were collected from the Ramaeshwaram sea shore of Bay of Bengal. The proteins were extracted from the sponge sample by Schroder and Ely method using β-Merkaptoethanol and Triton X-100 [10]. The sponge was cut into small pieces, and then weighed as much as 10 g, crushed in a blender using a solvent buffer A (Tris-HCl 0.1 M pH 8.3, 2 M NaCl, 0.01 M CaCl2, 1% β-merkaptoetanol, Triton X-100 0.5%), filtered with filter paper and the obtained filtrate was freeze thawed 2-3 times and then centrifuged at 12,000 rpm, 4°C, for 30 minutes. Furthermore, the supernatant was stored in the refrigerator prior to testing various activities.

Quantification of Sponge Protein

Protein content of sponge was determined by Barford method (Biorad reagent) [4] using Bovine Serum Albumine (BSA) as standard. The absorbance is measured at 595nm to estimate the protein content in the sample.

Neuroprotective Assays

Hydrogen Peroxide Scavenging Activity:

The hydrogen peroxide radical scavenging property of the sponge extract was determined using the methodology by [11, 12, 13]. PBS (pH 7.4) is used for the preparation of 40mM H_2O_2 solution, various concentrations of sponge extract is added in 0.6 ml of the prepared H_2O_2 solution. The absorbance was measured at 230nm which was determined after an incubation of period of 10 minutes. The hydrogen peroxide scavenging activity is calculated using the formula:
$\% \text{ Scavenged } [H_2O_2] = [(A_C - A_S)/A_C] \times 100$
Where A_C is the absorbance of the control and A_S is the absorbance of the extract or standards with H_2O_2.

Nitric Oxide Scavenging Activity

The nitric oxide scavenging activity for the sponge extract is performed by slight modifications in the method followed by Sreejayan and Rao et al by diluting a stock solution of 4g/ml of the crude extract [14, 15]. Ascorbic acid was used as standard and the absorbance was measured at 546 or 550 nm. The percentage nitrite radical scavenging activity of the sponge extracts and Ascorbic acid were calculated using the following formula:

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 7760 - 7771
Received 16 February 2021; Accepted 08 March 2021.

Nitric oxide scavenged (%) = $[(A_{control} - A_{test})/ A_{control}] \times 100$
Where $A_{control}$ = absorbance of control sample and A_{test} = absorbance in the presence of the samples of extracts or standards.

Superoxide Radical Scavenging Activity
Superoxide Radical Scavenging assay is based on the ability of the sample to inhibit the phytochemical reduction of NBT (Nitro Blue Tetrazolium) [16, 17] carried out with slight modifications in the method followed by Martinez et al. The total volume of the reaction mixture consists of 0.05 M PBS (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, NBT (75 µM) and 1.0 ml of test sample with different concentration as mentioned in the previous assays was incubated for 20 minutes. The absorbance was measured after incubation in the presence of fluorescent light (725 lumens, 34 W) at 560 nm. Ascorbic acid was used as the standard. The inhibition of superoxide anion was estimated by the equation:
% inhibition = $[(A_0 - A_S)/ (A_0)] X 100$
Where, A_0 is the absorbance of the control, and A_s is the absorbance of the tested sample.

Free Radical Scavenging Activity Assays
DPPH Assay
The DPPH radical-scavenging activity was performed as per the procedure followed by Qiao with modifications [18, 19, 20]. 0.2 ml of DPPH (0.4 mM) in dehydrated alcohol was mixed with 1.0 ml of the sample and 2.8 ml of distilled water, shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm and the scavenging activity of DPPH was measured using the formula:
DPPH radical scavenging activity (%) = $\{1 – [(A_1 – A_2)/ (A_0)]\times 100\}$
Where, A_0 is the absorbance of the control (water instead of the sample), A_1 is the absorbance of the sample and A_2 is the absorbance of the sample only (water instead of DPPH).

Hydroxyl Radical Scavenging Activity
The hydroxyl radical-scavenging activity was determined based on the method of Yu [24] with some modifications. The samples were taken in various concentrations as mentioned above to which 1ml of 9 mM $FeSO_4$ and salicylic acid-ethanol solution. 1 ml of 8.8 mM H_2O_2 was added to the mixture and the reaction was carried out at 37 ◦C for 1 h. The absorbance was made at 510 nm and the scavenging activity of the hydroxyl radical was calculated by the following equation:
Hydroxyl radical scavenging activity (%) = $\{1 – [(A_1 - A_2)/ A_0 \times 100\}$ (2)
Where, A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample and A_2 is the absorbance of the sample only (salicylic acid–ethanol solution instead of $FeSO_4$ and H_2O_2 solutions).

Fe^{3+} Reducing Antioxidant Power Assay
The FRAP assay of the sponge extract was determined based on the method proposed by Oyaizu (1986) with slight modification [21, 22, 23] Various concentrations of sponge extract dissolved in 1 ml of distilled water, 2.5 ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% [K3Fe(CN)6]. The reaction mixture was incubated for 20 minutes at 50˚C. The aliquots is then added with 2.5 ml of TCA. The upper layer of the mixture is diluted with 2.5 ml of distilled water and $FeCl_3$ (0.5 mL, 0.1%). The absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates an increase of reduction capability.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 7760 - 7771
Received 16 February 2021; Accepted 08 March 2021.

Cytoprotective Assay

The MTT assay was performed in (L929 cell line) were as the anticancer assay was performed in (C6 cell line) the according to the procedure followed by Mosmann.T, 1983[25, 26] employed for the assessment of the cell viability and also the aniti-cancer property of the sponge extract in 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The cells when treated with MTT solution (50 µL) in the concentration of 1 mg/mL incubated at 37°C for 48 hours. Finally, 150 µL of DMSO was added to the viable cells which were capable of producing the formazan crystals. The absorbance was measured at 570 nm.

Scratch Assay

Wound healing assay was performed with modifications in the method proposed by Lampugnani in the year 1999 [27, 28]. The cells, L929-Mouse Fibroblast Cell line (NCCS, Pune)are grown in DMEM supplemented with 10% FBS and other culture conditions. The grow cells are let in the environment for additional 24 to 48 h (or the time required if different cells are used). A gap is made by scratching the cell surface with a sterile needle. The mobility of the cells towards each other is quantified by measuring the gap distance using software such as Image J and each experimental group should be repeated multiple times.

RESULTS

The collected sponge was identified to be *Callyspongia.sp* which was extracted for proteins using β-mercaptoethanol and triton X-100 and were found to have 1882.513 µg/ml protein content. The extract was subjected to Neuroprotective and free radical scavenging property tests.

The graph (Fig:1) represents the results of the hydrogen peroxide radical scavenging activity of the sponge extract. It was observed that the % inhibition of the scavenging activity of hydrogen peroxide radicals of the extract increases with the increase in concentration of the sample, thus when compared to standard, the sample values was found be high.

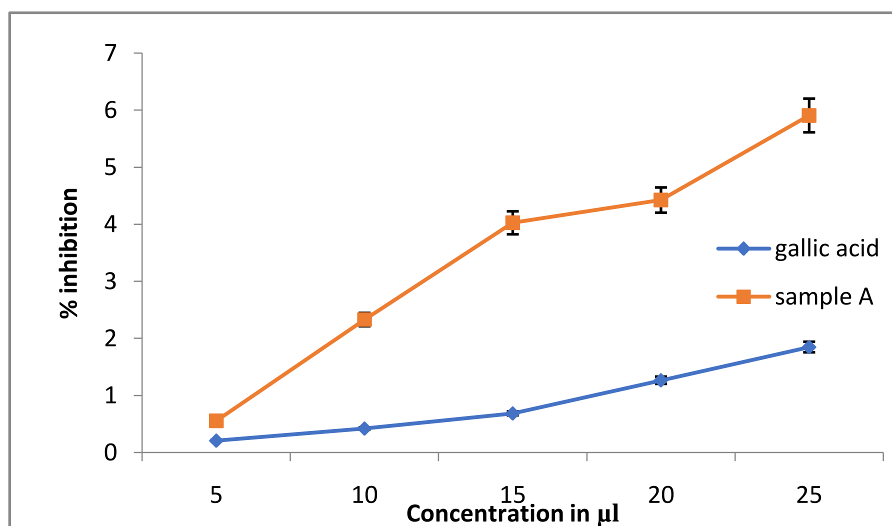


Fig: 1 Hydrogen Peroxide Radical Scavenging Activity

The nitric oxide scavenging activity of the nitric oxide generated from $Na_2[Fe(CN)_5NO]$ was identified by the Greiss reagent. When sodium nitro prusside dissolved in distilled water at physiological pH spontaneously generates nitric oxide which when interacts with the atmospheric oxygen releases nitrite ions and this can be estimated with the help of Greiss reagent. It is

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 7760 - 7771
Received 16 February 2021; Accepted 08 March 2021.

observed that there is an increase in inhibition percentage simultaneously with the increase in sample concentration. Here ascorbic acid used as standard.

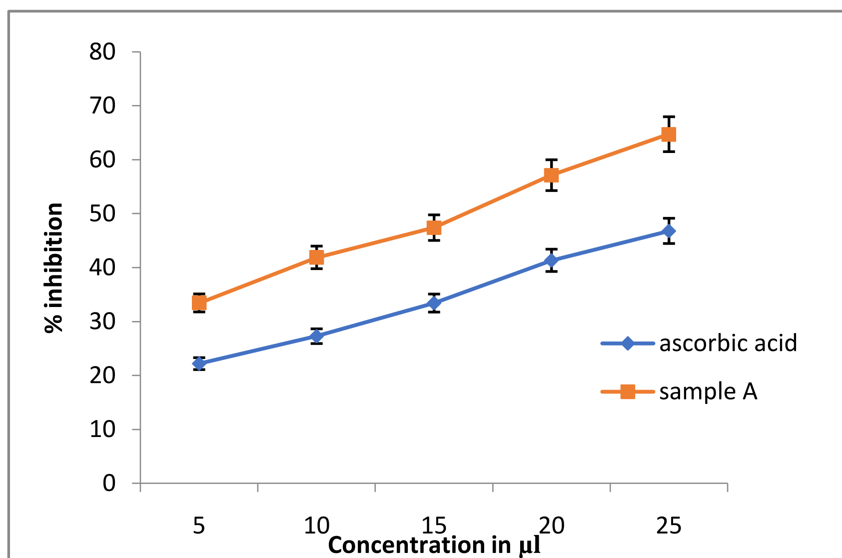


Fig: 2 Nitric Oxide Radical Scavenging Activity

The superoxide radical scavenging activity was determined based on the inhibition of the photochemical reduction of Nitro Blue Tetrazolium by the sponge extract. For this assay BHA (ButylatedHydroxy Anisole) was used as standard. It was observed that the samples showed less activity when compared to the standard at low concentration say 100 µl but as the concentration of the sample increases there was an increase in the percentage inhibition.

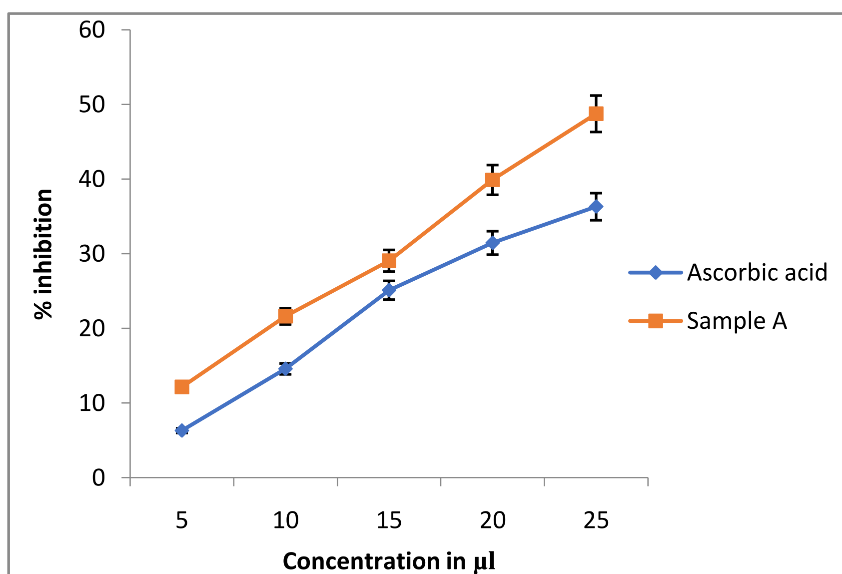


Fig: 3 Superoxide Radical Scavenging Activity

The scavenging of the free radical 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) was by reducing the protons after the reaction with the sample. The extract from marine sponge exhibited an increased inhibition activity against the DPPH free radical with the increase in the concentration. Thus the standard used was ascorbic acid and the value of the sponge extract was found to be higher than that of the standard values respectively.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 7760 - 7771
Received 16 February 2021; Accepted 08 March 2021.

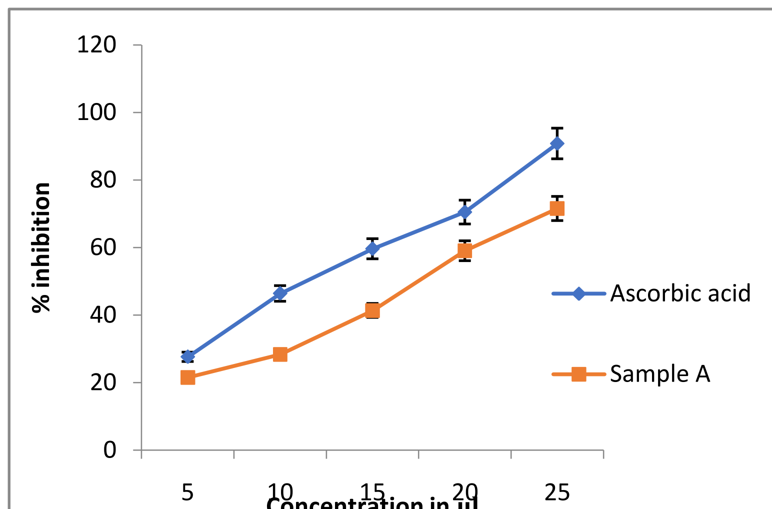


Fig: 4 DPPH Scavenging Activity

The Hydroxyl radical scavenging activity of the extract obtained indicates the inhibiting ability of the hydroxyl ions generated. When the sponge extract at various concentrations is added to the $FeSO_4$ and salicylic acid solution in the presence of H2O2 it was found to inhibit the generation of the hydroxyl radicals efficiently when compared to the standard BHT.

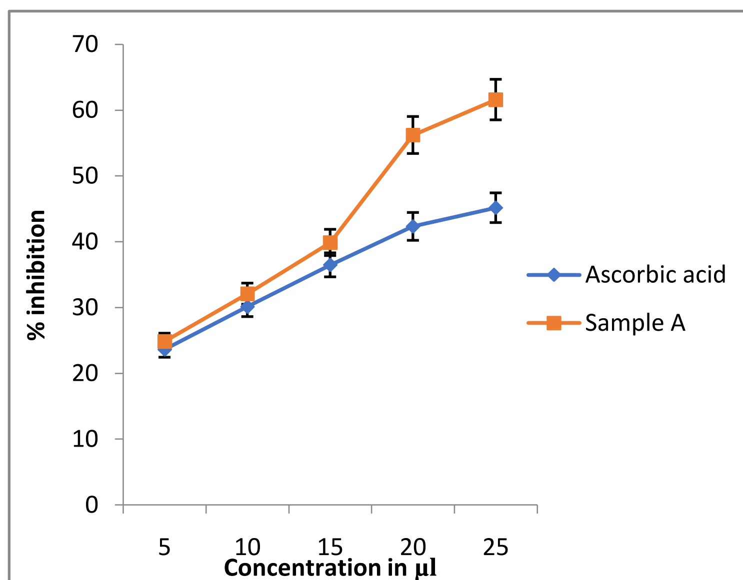


Fig: 5 Hydroxyl Radical Scavenging Activity

The FRAP assay was performed on the basis of the reduction in the ferric ion radicals based on the concentration of the sample. From the observations it was found that the reduction in the ferric ion radicals increases with the sample concentration. Thus the inhibition values obtained was more or less similar to values obtained for ascorbic acid which was used as standard.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 7760 - 7771
Received 16 February 2021; Accepted 08 March 2021.

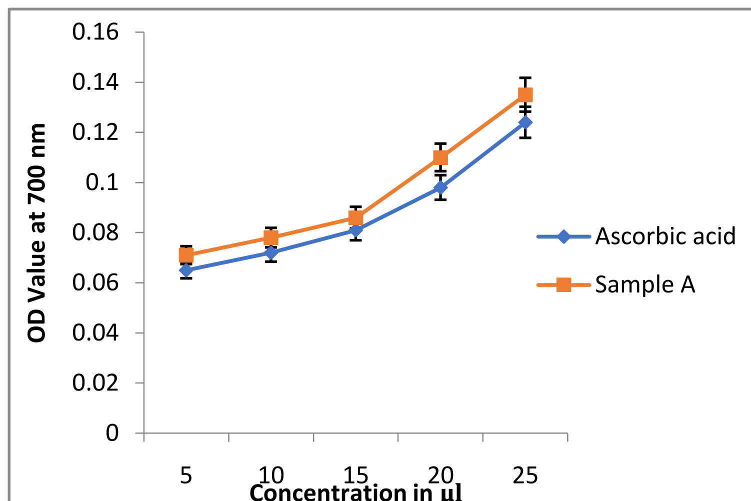


Fig: 6 Fe^{3+} Reducing Power Activity

In this study, Test Compound (Sample 1- sponge extract) is evaluated to check the Cytotoxicity Study on the 2 cell lines namely, L929 and C6. Extracellular reducing components such as ascorbic acid, cholesterol, alpha-tocopherol, dithiothreitol in the culture media reduces the MTT to formazan. In order to account this reduction, the same medium is used in control as well as test wells. The experiments were performed in duplicates. The obtained values were plotted in graph.

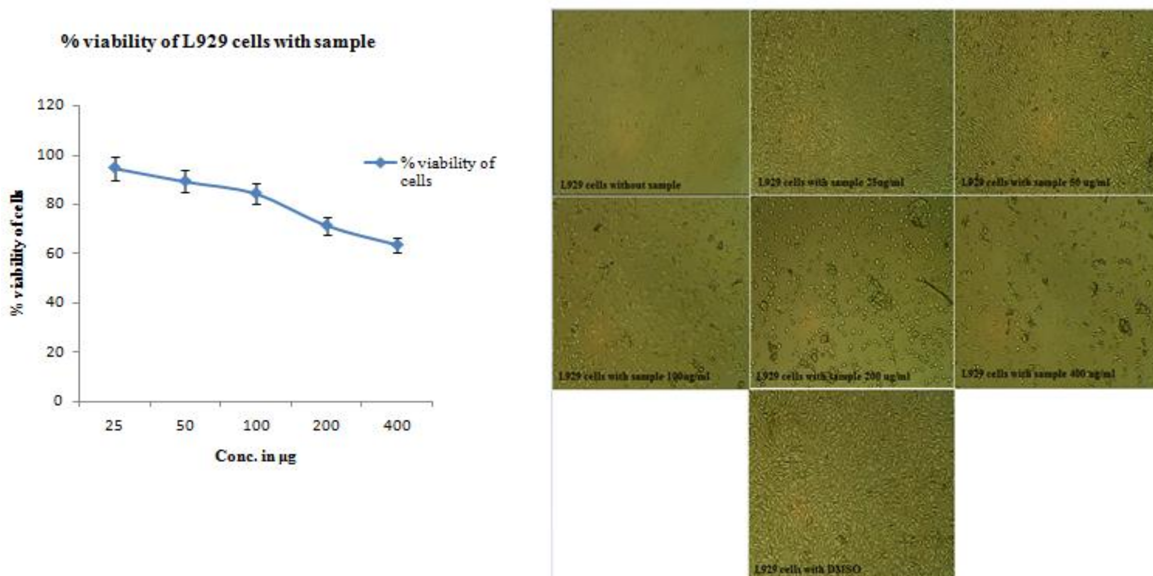


Fig:7 MTT Assay

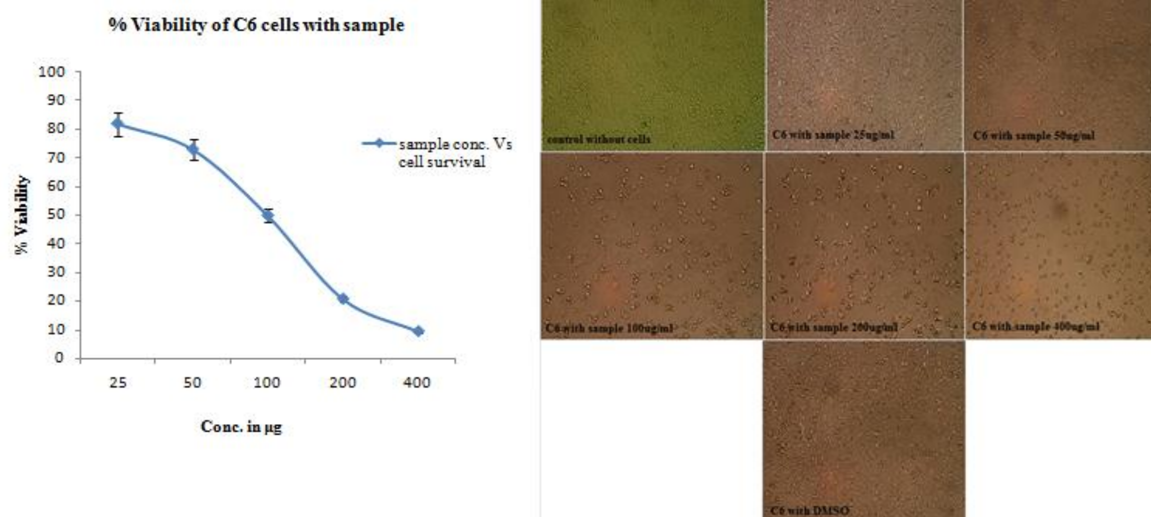
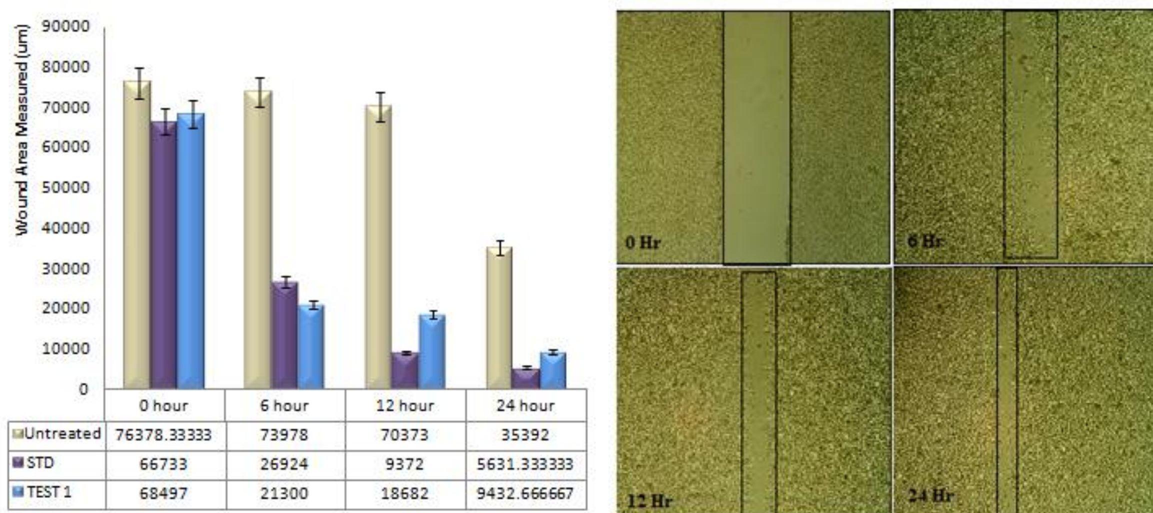


Fig: 8 Anti-Cancer Activity

The results of the scratch assay were represented below. The effectiveness of the sample was compared with that of the standard. According to the results obtained it was found that the protein sample exhibits an excellent healing property which was examined at various time intervals like 0, 6, 12 and 24 hours respectively.



	0 hour	6 hour	12 hour	24 hour
Untreated	76378.33333	73978	70373	35392
STD	66733	26924	9372	5631.333333
TEST 1	68497	21300	18682	9432.666667

Fig: 9 Wound healing Assay

DISCUSSION

The ability of the extract to scavenge hydrogen peroxide was attributed by the presence of the phenolics content are the hydrogen peroxide is reduced to water by donating electrons [29]. Thus the effective scavenging of H2O2 is done by the extract using the method proposed by Ruch [30]. Nitric oxide is an extensive free radical moiety found distributed in organs and tissues which are supposed to play a vital role as neurotransmitter in CNS [31]. The aim to obtain the extract from

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 7760 - 7771
Received 16 February 2021; Accepted 08 March 2021.

marine sponge is to inhibit the over production of nitric oxide which is a toxic intermediate produced from the products nitrite and nitrate in the presence of oxygen and hence these toxic unstable intermediates are responsible for many biological disorders [32].
One among the harmful reactive oxygen species was the superoxide ion which has deleterious effect on the cellular components of a living system. The superoxide ions are capable of initiating lipid per oxidation indirectly by the generation of singlet oxygen [33]. Thus the scavenging activity of the sponge extract in the increasing concentration depicts its scavenging ability by inhibiting the lipid oxidation.
The DPPH readily gets scavenged in the presence of an antioxidant. The presence of phenolics and flavonoids in the compound indicates their ability to scavenge the DPPH radical to 1,1-diphenyl-2-picrylhydrazine. The rate of scavenging activity of DPPH depends on the phenolics which contribute electrons / addition of hydrogen atoms [34] and also the flavonoids which possess high antioxidant activity in nature due to the presence of double bonds in the C-rings [35, 36]. The DPPH radical scavenging activity of the sponge extract was directly proportional to the increasing concentration of the extract.
One among the most important oxygen species Hydroxyl radical which is responsible for lipid per oxidation and several other biological damages. These radicals are capable of degrading deoxyribose into fragments when they are formed in free solutions [37]. The sponge extract inhibited 91.02% of the hydroxyl radical at a concentration of 500µl when compared with the standard BHT it was 95.01% in the same concentration which is expressing a little variation. On comparing with the earliest report it is found that the sponge extract exhibited a good the inhibition capacity on comparing with other compounds.
In FRAP assay the antioxidants were used for identifying the reductants in the colorimetric method of redox-linked reactions, with the easily reduced oxidant systems. The basic principle behind the FRAP assay was that in the presence of antioxidant or a chelating agent, the formation of the complex is disturbed as a result the red colour of the complex is decreased [37, 38]. The reduction in colour is measured spectrophotometrically at 700nm and the values are plotted in a graph in order to obtain a dose dependent curve showing that the sponge extract values are marginally similar to that of the standard ascorbic acid.
MTT assay is a main analysis to determine the safe dose of the bioactive extract. In vitro cytotoxicity activity of a bioactive molecule is exhibited by cell culture models. MTT when added to insoluble formazan crystals, which, when dissolved in a suitable solvent, exhibit a purple colour, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570 nm [38]. The observations of the study by ELISA Reader exhibits that when the sample tested for cytotoxic effect in normal L929 cells suggested that the experimental sample after 48 hrs of treatment was found to be non- toxic and 90% viable cells with an IC 50 value of 90.33 µG/mL compared to DMSO as positive control. While the same experimental sample when treated against cancerous C6 cell line it exhibited anti-cancer property with an IC50 value of 119.45 uG/mL over Camptothecin as positive control.
In a particular scratch wound healing test, a "wound space" in a cell monolayer is made by scratching, and the "healing" of this space through cell migration towards the center of the gap is monitored and often quantified. Factors that alter cell motility and / or growth can cause the rate of "healing" of the gap to increase or decrease [39]. However, according to Pozzolini a qualitative wound healing score was assigned based on the observation of increased cell density in the scratches instead of a quantification of wound restriction [40]. This test is simple, inexpensive, and the experimental conditions can be easily adjusted for different purposes. [41].

CONCLUSION

The present in vitro study proves that the protein sample extracted from marine sponge has a potential activity against neurodegeneration with an effective viability of cells and wound healing property, also in addition the extract exhibits a good anti-cancer activity against C6 cell line. The molecular mechanism and the gene regulation studies can be made in future.

REFERENCES

[1] Kim, G. H., Kim, J. E., Rhie, S. J., & Yoon, S. (2015). The role of oxidative stress in neurodegenerative diseases. *Experimental neurobiology*, *24*(4), 325-340.

[2] Staniek, K., &Nohl, H. (1999). H2O2 detection from intact mitochondria as a measure for one-electron reduction of dioxygen requires a non-invasive assay system1. *BiochimicaetBiophysicaActa (BBA)-Bioenergetics*, *1413*(2), 70-80.

[3] Abele, D., &Puntarulo, S. (2004). Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *138*(4), 405-415.

[4] Jones, C. G., Hare, J. D., & Compton, S. J. (1989). Measuring plant protein with the Bradford assay. *Journal of chemical ecology*, *15*(3), 979-992.

[5] Halliwell, B., Gutteridge, J. M. C., & Cross, C. E. (1992). Free radicals, antioxidants, and human disease: Where are we now? *The Journal of Laboratory and Clinical Medicine*, *119*(6): 598-620.

[6] Pietta, PG. (2000). Flavonoids as antioxidants. Journal of Natural Products 63: 1035-1042.

[7] Uttara, B., Singh, A.V., Zamboni, P., &Mahajan, R.T. (2009). Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. Current Neuropharmacology, 7: 65-74.

[8] Thomas, T.R.A., Kavlekar, D.P., &Lokabharathi, P.A. (2010) Marine Drugs from Sponge-Microbe Association—A Review. *Marine Drugs.* 8: 1417-1468, 2010.

[9] Elliott, G.R.D., & Leys, S.P. (2010) Evidence for glutamate, GABA and NO in coordinating behaviour in the sponge, Ephydatiamuelleri (*Demospongiae, Spongillidae*).*Journal of Experimental Biology*. 213: 2310-2321.

[10] Salehi, A., Patong, R., & Ahmad, A. (2014). Isolation and characterization of some kind bioactive proteins sponge as antibacterial agent. *International Journal of Scientific & Technology Research*, *3*(2), 233-236.

[11] Ruch, R. J., Cheng, S. J., &Klaunig, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, *10*(6), 1003-1008.

[12] Keser, S., Celik, S., Turkoglu, S., Yilmaz, O., &Turkoglu, I. (2012). Hydrogen peroxide radical scavenging and total antioxidant activity of hawthorn. *Chem J*, *2*(1), 9-12.

[13] Leelavathi, M. S., & Prasad, M. P. (2016). A study on antioxidant properties of seaweeds by hydrogen peroxide assay. *Int Res J EngTechnol*, *3*, 922-6.

[14] Rao, M. N. A. (1997). Nitric oxide scavenging by curcuminoids. *Journal of Pharmacy and Pharmacology*, *49*(1), 105-107.

[15] Boora, F., Chirisa, E., &Mukanganyama, S. (2014). Evaluation of nitrite radical scavenging properties of selected Zimbabwean plant extracts and their

phytoconstituents. *Journal of Food Processing*, *2014*.

[16] Martinez-Tome, M., Jimenez, A. M., Ruggieri, S., Frega, N., Strabbioli, R., & Murcia, M. A. (2001). Antioxidant properties of Mediterranean spices compared with common food additives. *Journal of Food Protection*, *64*(9), 1412-1419.

[17] Awah, F. M., Uzoegwu, P. N., Oyugi, J. O., Rutherford, J., Ifeonu, P., Yao, X. J., ... &Eze, M. O. (2010). Free radical scavenging activity and immunomodulatory effect of Stachytarphetaangustifolia leaf extract. *Food Chemistry*, *119*(4), 1409-1416.

[18] Qiao D, Ke C, Hu B, Luo J, Ye H, Sun Y, Yan X, Zeng X. Antioxidant activities of polysaccharides from Hyriopsiscumingii. Carbohyd. Polym. . 2009;78:199–204.

[19] Zhu, W., & Zhang, Z. (2014). Preparation and characterization of catechin-grafted chitosan with antioxidant and antidiabetic potential. *International journal of biological macromolecules*, *70*, 150-155.

[20] Ting, Z., Yina, F., Guanghua, M., Weiwei, F., Yanmin, Z., Ye, Z., ...&Xiangyang, W. (2017). Purification, characterization and antioxidant activities of enzymolysis polysaccharide from Grifolafrondosa. *Iranian journal of pharmaceutical research: IJPR*, *16*(1), 347.

[21] Oyaizu M (1986). Studies on product of browning reaction prepared from glucose amine. J. Nutr., 44: 307-315.

[22] Büyükokurolu ME, Gülçin (2009). In vitro antioxidant and antiradical properties of Hippophaerhamnoides L. Phcog. Mag., 4: 189-195.

[23] Koksal, E., Bursal, E., Dikici, E., Tozoglu, F., &Gulcin, I. (2011). Antioxidant activity of Melissa officinalis leaves. *Journal of Medicinal Plants Research*, *5*(2), 217-222.

[24] Yu, L., Haley, S., Perret, J., & Harris, M. (2004). Comparison of wheat flours grown at different locations for their antioxidant properties. *Food Chemistry*, *86*(1), 11-16.

[25] Mosmann T. Rapidcolorimetric assay for cellular growth and survival: application to proliferation andcytotoxicity assays. J Immunol Methods 1983; 65:55–63.

[26] Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., &Kissel, T. (2003). In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials*, *24*(7), 1121-1131.

[27] Liang, C. C., Park, A. Y., & Guan, J. L. (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature protocols*, *2*(2), 329.

[28] Chen, Y. (2012). Scratch wound healing assay. *Bio-protocol*, *2*(5), e100-e100.

[29] Nabavi SM, Ebrahimzadeh MA, Nabavi SF and Jafari M (2008). Free radical scavenging activity and antioxidant capacity of EryngiumcaucasicumTrautv and Froripiasubpinnata. Pharmacologyonline, 3: 19- 25.

[30] Ebrahimzadeh, M. A., Nabavi, S. M., Nabavi, S. F., Bahramian, F., &Bekhradnia, A. R. (2010). Antioxidant and free radical scavenging activity of H. officinalis L. var. angustifolius, V. odorata, B. hyrcana and C. speciosum. *Pak J Pharm Sci*, *23*(1), 29-34.

[31] Singh, D., Mishra, M., Gupta, M., Singh, P., Gupta, A., &Nema, R. (2012). Nitric Oxide radical scavenging assay of bioactive compounds present in methanol Extract of Centellaasiatica. *Int J Pharm PharmSci Res*, *2*(3), 42-44.

[32] Menaga, D., Rajakumar, S., &Ayyasamy, P. M. (2013). Free radical scavenging activity of methanolic extract of Pleurotusflorida mushroom. *International journal of pharmacy and pharmaceutical sciences*, *5*(4), 601-606.

[33] Mandal, S., Hazra, B., Sarkar, R., Biswas, S., &Mandal, N. (2011). Assessment of the antioxidant and reactive oxygen species scavenging activity of methanolic extract of Caesalpinia crista leaf. *Evidence-Based Complementary and Alternative Medicine*, *2011*.

[34] Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. LebensonWissTechnol 28:25–30.

[35] Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free RadicBiol Med 20:933–956

[36] Kondo K, Kurihara M, Fukuhara K, Tanaka T, Suzuki T, Miyata N, Toyoda M (2000) Conversion of procyanidin B-type (catechin dimer) to A-type: evidence for abstraction of C-2 hydrogen in catechin during radical oxidation. Tetrahedron Lett 41:485–488

[37] Kumar, S., Kumar, D., SAROHA, K., Singh, N., &Vashishta, B. (2008). Antioxidant and free radical scavenging potential of Citrulluscolocynthis (L.) Schrad. methanolic fruit extract. *ActaPharmaceutica*, *58*(2), 215-220.

[38] Weecharangsan, W., Opanasopit, P., Sukma, M., Ngawhirunpat, T., Sotanaphun, U., &Siripong, P. (2006). Antioxidative and neuroprotective activities of extracts from the fruit hull of mangosteen (Garciniamangostana Linn.). *Medical Principles and Practice*, *15*(4), 281-287.

[39] Kobayashi, M., Kawase, T., Horimizu, M., Okuda, K., Wolff, L. F., &Yoshie, H. (2012). A proposed protocol for the standardized preparation of PRF membranes for clinical use. *Biologicals*, *40*(5), 323-329.

[40] Pozzolini, M., Millo, E., Oliveri, C., Mirata, S., Salis, A., Damonte, G., ...&Scarfì, S. (2018). Elicited ROS scavenging activity, photoprotective, and wound-healing properties of collagen-derived peptides from the marine sponge Chondrosiareniformis. *Marine drugs*, *16*(12), 465.

[41] Yarrow, J. C., Perlman, Z. E., Westwood, N. J., &Mitchison, T. J. (2004). A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. *BMC biotechnology*, *4*(1), 21.